Genetic Diversity in Black Howler Monkeys (*Alouatta pigra*) From Belize

ROXIE A. JAMES,[1,4]* PAUL L. LEBERG,[2] JOSEPH M. QUATTRO,[3] AND ROBERT C. VRIJENHOEK[4]
[1]*Department of Biology, Kean College of New Jersey, Union, New Jersey 07083-0411*
[2]*Department of Biology, University of Southwestern Louisiana, Lafayette, Louisiana 70504*
[3]*Department of Biology, University of South Carolina, Columbia, South Carolina 29208*
[4]*Center for Theoretical and Applied Genetics Rutgers University, New Brunswick, NJ 08903-0231*

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ABSTRACT To assess the level of genetic variation in a threatened black howler monkey (*Alouatta pigra*) population, we examined 36 allozyme loci and restriction fragment profiles of mitochondrial DNA (mtDNA). Mean heterozygosity at allozyme loci was only 0.021 and 5.6 percent of the loci were polymorphic. Analyses of mtDNA also revealed low genetic diversity compared with other primates. F-statistics revealed no significant genetic heterogeneity among troops within the Bermudian Landing preserve, but did indicate a deficiency of heterozygotes at one of the two loci. We explore several explanations for this result, which is unexpected in a socially structured primate. Low genetic diversity in this population may reflect its history of demographic bottlenecks. Am. J. Phys. Anthropol. 102:329–336, 1997.

Belizean black howler monkeys (*Alouatta pigra*, Lawrence, 1933) have undergone four population crashes in recent times. Devastating hurricanes swept through Bermudian Landing, Belize in 1931, 1954, and 1978 (Hartshorn, 1984; Bolin, 1981), and a yellow fever epidemic decimated Central American howlers in 1971 (Baldwin, 1976; Hartshorn, 1984). Each of these events reduced local populations to very low levels, affecting both the sizes and the behavioral dynamics of remnant troops (Baldwin, 1976; Bolin, 1981). Although remnant populations have recovered, troops remain small compared to other howler species such as Costa Rican golden mantled howlers (*Alouatta palliata*, Gray, 1849) in which troop size averages about 20 (Glander, 1980). Troop composition in black howlers varies but generally consists of one dominant adult male, one or two adult females, plus subadults and juveniles (mean troop size = 4.6, S.D. = 1.7).

When a population experiences a demographic bottleneck, genetic diversity is expected to decline depending upon the bottleneck population size and the subsequent rate of population growth (Chakraborty and Nei, 1977; Maruyama and Fuerst, 1985a,b;

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*Correspondence to: Roxie A. James, Department of Biology, Kean College of New Jersey, Union, NJ 07083.

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Nei et al., 1975; Leberg, 1992). A demographic bottleneck can result from either a reduction in the size of a population to a few individuals or from the establishment of a population by a small number of founders. Given the recent history of bottlenecks in the Bermudian Landing black howlers, we would expect low levels of heterozygosity and allelic diversity compared with larger more stable populations. Barring immigration, it would take a very long time to restore variability in a troop through accumulated mutation (Sirkkomaa, 1983).

Social structure in Belizean howler populations might affect levels of genetic diversity in and among troops. Long-term behavioral studies identified a number of troops, each of which constitutes a stable assemblage of individuals with respect to home range (Horwich, 1983a,b; Horwich and Lyon, 1990). Social structure which imposes deviations from random mating and reduces dispersal among local breeding units can have significant effects on the distribution and amount of genetic variation in mammalian populations (Bush et al., 1977; Chesser, 1991a,b; Melnick et al., 1984; Smouse and Neel, 1981). For example, social structure can result in genetic differences among troops, as is seen in the red howler (*Alouatta seniculus;* Pope, 1992). The effects of population bottlenecks on genetic diversity are also dependent on social structure. In a demic model with limited gene flow among subpopulations, demographic bottlenecks decrease genetic diversity within the subpopulations but increase genetic differences among them. However, in populations comprised of social groups of philopatic females and dispersing males, demographic bottlenecks decrease both within-group diversity and genetic differences among groups (Chesser, 1991a).

The primary purpose of this paper is to examine genetic diversity and population structure in Belizean black howlers (*Alouatta pigra*) using allozyme loci and mitochondrial DNA (mtDNA). Our assessment of genetic diversity within and among social groups should provide useful information to conservation biologists attempting to preserve this species, as well as insight into the effects of population bottlenecks on the evolution of socially structured populations of primates.

MATERIALS AND METHODS

Howler troops were studied in the Community Baboon Sanctuary (CBS), Bermudian Landing, Belize. These troops had been defined previously on the basis of behavioral studies (Horwich, 1983a,b). The CBS is an area of second-growth forest; the sanctuary is not isolated from nearby habitat supporting howler troops.

Tissue samples were collected from all black howlers occupying the Community Baboon Sanctuary, during January–February 1990. We used standard dart gun techniques (Cohen and Bree, 1978; Glander, 1991; Scott et al., 1976) to anesthetize juveniles and adults within their habitats. Darts were loaded with 25 mg of Telazol (Tiletamine hydrochloride and zolazepam hydrochloride, A.H. Robbins Co., Richmond, VA) per kg of body weight. Animals falling from trees were caught in a nylon mesh net. Once on the ground, we took a blood sample from the femoral vein of all individuals >6 months of age. Blood was held in a Vacutainer (Becton Dickinson, Co., Rutherford, NJ) syringe with the anticoagulant EDTA. A small notch was cut in the ear lobe to mark each individual, regardless of age. We placed the ear tissue in a sterile 1.5 ml microcentrifuge tube and froze it immediately on dry ice. The anesthetized monkeys were placed in burlap bags in the shade until they recovered from the anesthesia.

The blood samples were kept on water ice packs for up to 6 hours in the field. Each evening at the field station, the fresh blood samples were centrifuged at 1,500 rpm for 20 minutes to separate plasma, erythrocytes, and leukocytes. The erythrocyte subsample was washed three times in 0.9% saline and suspended in an equal volume of mannitol-glycerol-saline solution to prevent lysis (Melnick et al., 1984). All samples were packed on dry ice and held at $-20°C$ for up to 10 days prior to transport to Rutgers University.

In the laboratory, samples of red cells were thawed and lysed with an equal volume of ice-cold grinding buffer, Tris-EDTA-$MnCl_2$, pH 7.0. Plasma cells were lysed by

centrifugation (Harris and Hopkinson, 1978; Melnick et al., 1984). Extracts were loaded into preformed wells of 12% starch gels in the appropriate buffer (Table 1). Standard procedure for starch gel electrophoresis follows Harris and Hopkinson (1978) and Murphy et al. (1990). The designations used for gene loci follow those of Harris and Hopkinson (1978).

Allelic frequencies and tests for deviations from Hardy-Weinberg expectations were calculated using the computer program BIOSYS-1 (Swofford and Selander, 1981). Estimation of population structure were made using Wright's F-statistics (1965) with modifications following Nei (1977). One solitary male was not used in the estimation of the F-statistics because it could not be assigned to a troop. Because almost the entire population (all members >6 months of age) was sampled in terms of both troops and individuals, we did not use the approach of Weir and Cockerham (1984), which corrects for error due to incomplete sampling of demes and individuals. Following the suggestion of Chesser (1991a), we attempted to make separate estimates of the F-statistics for the adults, subadults, and juveniles; unfortunately, the number of individuals in the troops was too small to conduct this analysis.

We tested the significance of F_{IT}, the correlation of uniting gametes within individuals relative to that of the total population, F_{IS}, the average correlation of uniting gametes within individuals relative to that of a subdivision (troop, in our case), and F_{ST}, the correlation of uniting gametes within troops relative to that of the total population, using a randomization approach similar to that used by Pope (1992). The only deviation from her approach is that alleles, not genotypes, found in the 39 individual troop members, were randomly reassigned to individuals in the 10 troops. By randomly reassigning alleles to individuals and troops, rather than reassigning genotypes to troops, we could evaluate the significance of F_{IT}, as well as the other two components of genetic variance. This method was used to evaluate the significance of F-statistics for single loci, and mean values based on multiple loci; the results which these hypotheses test were not

TABLE 1. Proteins encoded by 36 putative gene loci in Belizian Alouatta pigra. *Enzyme systems are listed by tissue preparations that provided best resolution*

Protein	EC number	Code	Buffer[1]
Plasma			
Adenosine deaminase II	3.3.4.4	*Ada-I*	1
Aspartate aminotransferase	2.6.1.1	*Aat-I*	2
Carbonic Anhydrase	4.2.1.1	*Ca*	4
Creatine Kinase II	2.7.4.3	*Ck-II*	2
Esterase C	3.1.1.-	*Es-C*	4
Esterase D	3.1.1.-	*Es-D*	4
Glutamate Dehydrogenase	1.1.1.47	*Gdh*	2
Peptidase A	3.4.11	*Pepd-A*	4
Peptidase C	3.4.11	*Pepd-C*	4
Peptidase E	3.4.11	*Pepd-E*	4
Phosphoglucomutase	2.7.5.1	*Pgm-II*	4
Erythrocyte (1:10 dilution)			
Adenylate kinase	2.7.4.3	*Adk*	2
Catalase	1.11.1.6	*Cat*	2
Creatine kinase I	2.7.4.3	*Ck-I*	2
Malate dehydrogenase I	1.1.1.37	*Mdh*	4
Malic enzyme	1.1.1.37	*Me*	2
Phosphoglucose isomerase I	5.3.19	*Pgi-I*	6
Erythrocyte (undiluted)			
Adenosine deaminase I	3.5.4.4	*Ada-I*	1
Albumin		*Alb*	1
Esterase A	3.1.1.-	*Es-A*	4
Esterase B	3.1.1.-	*Es-B*	4
General protein I		*Gp-I*	1
General protein II		*Gp-II*	1
Glucose-2-dehydrogenase	1.1.1.8	*G2dh*	2
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6pdh*	3
αGlycerol phosphate dehydrogenase	1.1.1.43	*αGpd*	3
Hemoglobin		*Hb*	1
Lactate dehydrogenase A	1.1.1.27	*Ldh-A*	3
Lactate dehydrogenase B	1.1.1.27	*Ldh-B*	3
Mannose phosphate isomerase	5.3.1.8	*Mpi*	2
NADH-diaphorase	1.8.1.4	*Dia*	5
Peptidase B	3.4.11	*Pepd-B*	4
Peptidase D	3.4.13.9	*Pepd-D*	4
Phosphoglucomutase I	2.7.5.1	*Pgm-I*	4
Purine nucleoside phosphorylase	2.4.2.1	*Pnp*	7
Superoxide dismutase	1.15.1.1	*Sod*	3

[1] Buffers: 1, Tris-Boric Acid-EDTA (TBE 8.7); 2, Tris-Citric Acid (TC 6.8); 3, Tris-maleic acid-EDTA (TME 7.4); 4, Aminopropylmorpholine (AP 6.0); 5, Tris-citric acid (TC 6.0); 6, Tris versene borate (TVB 8.0); 7, Lithium hydroxide-boric acid (LHB 7.2). Buffers are described in Harris and Hopinson (1978) and Murphy et al. (1990).

*TABLE 2. Troop size (N), sample size (n), frequencies of common alleles and numbers of observed and expected heterozygotes (H_o and H_e) at two diallelic loci (*Pgi-1, Pnp*) in Bermudian Land troops of* Alouatta pigra

Troop	N	n	*Pgi-I* Freq.	H_o	H_e	*Pnp* Freq.	H_o	H_e
Fig tree	4	3	0.67	0	1.3	0.50	1	1.5
Wade pasture	5	3	0.67	0	1.3	0.50	1	1.5
Cashew	4	3	0.33	0	1.3	0.83	1	0.8
Della	7	7	0.64	3	3.2	0.57	2	3.4
Y	4	4	0.75	2	1.5	0.63	3	1.9
Northern A	5	3	0.37	1	1.4	0.37	2	1.4
A	4	4	0.63	1	1.9	0.25	2	1.5
Peninsula	6	6	0.83	2	1.7	0.75	3	2.3
Baptist	3	3	0.33	2	1.3	0.50	3	1.5
School	4	3	0.50	1	1.5	0.83	1	0.8
Mean[1]	4.6	3.9	0.60	1.5	1.9	0.58	2.0	1.9

[1] Means weighted by sample size of troops.

different from those obtained from more traditional tests of the significance of F-statistics (i.e., Workman and Niswander, 1970). Five thousand randomizations of the data were used to assess statistical significance at the 0.05 level.

Mitochondrial DNA variation was revealed by restriction analysis using 13 endonucleases (AVAI, AVAII, BAMHI, BGLII, ECORI, ECORV, HINCII, HINDIII, PVUII, PSTI, SCAI, XBAI, XHOI). Ear tissue was used in this analysis so mtDNA genotypes were available for all 47 individuals inhabiting the CBS, not just those over >6 months of ages used in the allozyme survey. The specific conditions of DNA extraction, restriction enzyme digestion, electrophoresis, and DNA transfer and visualization are described by Lansman et al. (1981) and Southern (1975). A heterologous mtDNA probe was isolated from the whole liver of a red howler monkey. The probe DNA was labeled by random primed incorporation of dioxigenin-labeled deoxyuridine-triphosphate (Genius, BMB). The number of nucleotide substitutions, and the divergence of between DNA sequences, was estimated for haplotypes with different patterns of restriction fragments (Nei, 1987; Nei and Li, 1979).

RESULTS

Allozyme variation

Based on comparison with human isozyme patterns (Harris and Hopkinson, 1978), we inferred that the 24 proteins surveyed (Table 1) are encoded by 36 putative gene loci. Only two loci (*Pgi-I* and *Pnp*) were polymorphic in our sample of *A. pigra* (Table 2). Polymorphism occurred in all ten troops, so the percentage of polymorphic loci (P) was 5.6%. Mean heterozygosity (H) varied from troop to troop, but averaged 0.021.

TABLE 3. F-statistics at polymorphic gene loci in Bermudian Landing troops of Alouatta pigra

Locus	F_{IT}	F_{ST}	F_{IS}
Pgi-I	0.436*	0.116	0.362*
Pnp	0.056	0.135	−0.091
Mean	0.246	0.125	0.138

* $P < 0.05$.

There was no significant genetic differentiation among the ten troops; estimated F_{ST} for the two polymorphic loci were not significantly different from zero (Table 3). Likewise, average F_{IT} and F_{IS} were not different from zero; however, both F_{IS} and F_{IT} were larger than zero for *Pgi-I* locus (Table 3). The large F_{IS} and F_{IT} values for this locus were due to excess homozygosity within troops (Table 2). This high homozygosity was not due to a scoring bias against *Pgi-I* heterozygotes, as every individual was examined electrophoretically at least twice, and heterozygotes were unequivocal. The presence of an enzymatically silent *Pgi-I* allele could be responsible for the high homozygosity observed at this locus, but we observed no apparent homozygotes for a silent allele. All individuals produced a *Pgi-I* phenotype on the gels.

mtDNA diversity

Restriction enzymes with four base-pair recognition sequences generated 25 to 30

TABLE 4. A partial survey of studies reporting two indicies of genetic variation, the percentage of polymorphic loci (P%) and average multiple-locus heterozygosity (H) in primates

Population	Sample size	Number troops	Number loci	P%	H	Ref.[1]
Alouatta pigra. Belize	41	10	36	5.6	0.021	1
A. seniculus, Venezuela	137	18	29	32.7	0.099	2
Macaca senica, Sri Lanka	131	16	32	34.3	0.078	3
M. fuscata, Japan	1642	33	32	9.2	0.013	4
M. fuscata, Japan	35	01	33	18.2	0.044	5
M. fuscata yakui, Yaku Island	29	15	29	00.0	0.000	6
M. fasicularis, Indonesia	456	29	33	12.2	0.038	7
M. mulatta, Pakistan	189	05	35	14.3	0.055	9
Theropithecus gelada, Ethiopia	213	04	34	7.4	0.013	10
Papio hemadryas, Ethiopia	414	05	34	31.4	0.042	11
P. anubis, Ethiopia	40	02	34	31.4	0.032	11
Cercopithecus aethiops, Ethiopia	124	07	23	17.4	0.056	12
Homo sapiens, Caucasian			104	23.0	0.063	13

[1] References: 1, present study; 2, Pope (1992); 3, Shotake and Santiapillai (1982); 4, Nozawa et al. (1982); 5, Hayasaka (1987); 6, Nozawa et al. (1977); 7, Kawamoto et al. (1984); 8, Ishimoto (1973); 9, Melnick et al. (1984); Melnick (1987); 10, Shotake and Nozawa (1984); 11, Shotake (1981); 12, Turner (1981); 13, Harris and Hopinson (1978).

fragments per individual and produced fragment profiles that were uninterpretable. We limited our analysis to ten enzymes that recognize six base-pair sequences, two that recognize degenerate six base-pair sequences, and one that recognizes a degenerate five base-pair sequence. The sample of 47 black howler monkeys revealed two mtDNA haplotypes based on the presence or absence of a HINDIII restriction site. In total, 46 individuals exhibited the common profile (PIG1) and a single individual exhibited the variant profile characterized by an extra HINDIII restriction site (PIG2). No mtDNA variability was observed for the remaining 12 enzymes in *A. pigra*.

DISCUSSION

Levels of allozyme variation in the Bermudian Landing population of Belizean black howler monkeys are low relative to that found in other primates (Table 4). Mean heterozygosity for 36 allozyme loci was 0.021. Other primates and mammals in general tend to have much higher levels of heterozygosity. Pope (1992) found high levels of variation in Venezuelan populations of the red howler *A. seniculus*. Ten out of 29 gene loci were polymorphic ($P = 32.7$), and heterozygosity ranged from 0.057 to 0.135, with a mean H of 0.099. High levels of heterozygosity are also found in Asian populations of *M. senica, M. fuscata, M. fasicularis,* and *M. mulatta,* and African populations of *P. hemadryas, P. anubis* and *C. aethiops* (Table 4). Low levels of allozyme variation have been reported for some island populations of primates. An island population of the Japanese macaque, *M. fuscata yakui*, had no detectable heterozygosity at 29 putative gene loci, but mainland populations of this species also had relatively low heterozygosity, mean H = 0.013 (Nozawa et al., 1977, 1982). Island populations of the rhesus macaque, *M. mulatta*, also had low heterozygosity relative to mainland populations (Melnick, 1988). Founder events, severe bottlenecks, and continuous genetic drift in small island populations are likely to result in reduced variability relative to mainland populations (Soulé, 1973).

Relative to other primates, the mitochondrial DNA diversity of the Bermudian Landing black howler population also was very low (the percent sequence divergence $P = 0.2$). In a review of mtDNA surveys of eight species of *Macaca* and hominoids, Melnick and Hoelzer (1993) report P values of 0.9–6.0. Although our estimate of P is low compared to estimates from other primates, the results of this comparison is problematic. Sequence divergence is expected to increase with geographic isolation and many of the studies reported by Melnick and Hoelzer (1993) were conducted over a broader geographic scale than our study of black howlers. However, even in a study of social groups of toque monkeys (*M. sinica*) that regularly exchange males, there was substantially more sequence divergence

(P = 3.1) than was observed in black howlers (Hoelzer et al., 1994).

More interindividual differences were noted at the allozyme loci than for the mtDNA, but it is difficult to compare relative levels of genetic diversity because these genetic systems differ in several fundamental ways. Unlinked allozyme loci represent independent genetic markers while mtDNA restriction sites are linked because the molecule is transmitted as a single locus without recombination. Additionally, allozyme loci generally exhibit Mendelian inheritance, but mtDNA generally exhibits maternal transmission. Because of these differences, the effective population size of mtDNA is smaller than the effective population size for nuclear genes such as allozymes (Wilson et al., 1985; Avise et al., 1988). If the population of Black Howlers at Bermudan Landing has been severely reduced in the past, the relatively small effective size of mtDNA means that diversity at this locus would be lost more rapidly than allozyme diversity. Although the almost complete absence of mtDNA diversity in *A. pigra,* taken along with the low levels of allozyme variation, is consistent with the hypothesis that the Bermudian Landing population was drawn through one or more recent severe bottlenecks, we cannot exclude the possibility that low genetic diversity is characteristic of black howler monkey populations, in general. Samples of *A. pigra* from other localities would be necessary to refute the latter hypothesis. A general finding of naturally low levels of variability in other populations would diminish the need for management programs aimed at elevating levels of genetic diversity in locally threatened populations, such as the Bermudian Landing black howlers.

Analysis of population structure with Wright's F-statistics suggests there is no differentiation among black howler troops. For one of the two loci, there was also a deficiency of heterozygotes. These observations differ dramatically from those made for troops of red howlers (*A. seniculus*) which exhibit excess heterozygosity within populations and significant genetic differentiation among troops (Pope, 1992). Excess heterozygotes within troops with a lack of intertroop differentiation has also been observed in other primates (Melnick et al., 1984).

In red howler monkeys, females typically remain in their natal troops or occasionally disperse to establish new troops, while dispersing males often immigrate to and reproduce in troops in which they were not born (Pope, 1992). Under this system of social structure, excess heterozygosity within groups and significant differentiation among troops, is the theoretical expectation (Chesser, 1991a; Pope, 1992). It is interesting to speculate why black howlers deviate from these expectations. Past demographic bottlenecks would reduce genetic differentiation among troops (Chesser, 1991a); however, they do not explain the heterozygote deficiencies occurring in most troops. One potential explanation for the heterozygote deficiencies and lack of spatial structure may simply result from our inability to estimate Wright's F-statistics precisely due to a small number of polymorphic loci in the black howler troops. Interlocus variation in estimates of F-statistics are often the major source of the variation in these estimates (Nei, 1978). With only two polymorphic loci, it is possible that our measurements of F_{IS}, F_{IT}, and F_{ST} poorly estimate the true values of these parameters.

Deviation of the F-statistics from the expectations derived by Chesser (1991a,b) may also result from the demographic bottlenecks the Bermuda landing population has experienced. These exceptions assume populations are at a demographic equilibrium with nonoverlapping generations. This assumption clearly does not apply to the black howler troops we examined, given the recent history of population declines in the CBS. In the most recent documented bottleneck, howler troops were thought to be drastically reduced by a hurricane in 1978 (Bolin, 1981). Following the hurricane, most of the howler troops were comprised of only a single pair of adults. This unusual social structure was assumed to be related to the merging of remnants of troops devastated by the hurricane (Bolin, 1981), suggesting the demographic and genetic structure of the population was disturbed as recently as 1978. Simulations of Chesser (1991a) suggest that a population would reach equilibrial levels

of heterozygosity excess within troops, as well as among troop differentiation, within six generations. Although we have insufficient data to precisely quantify generation length in black howlers, marked individuals do not reproduce until 4–5 years of age, and one female was still reproductively active at 20 years of age. We collected our samples 12 years after the population was greatly reduced by the hurricane. Thus, it is likely that sufficient time has occurred for the population to reach equilibrial conditions with regard to Wright's F-statistics.

Another possibility for the differences between the genetic structure of black howlers and that of red howlers (Pope, 1992) may result from differences in dispersal patterns of these two species. Pope (1992) found that essentially no female successfully immigrated to an established troop after dispersing from its natal troop. We are just beginning to accumulate data on dispersal of marked individuals in the Bermudian Landing population of black howlers (Horwich and James, in prep.), but it appears that some females successfully move among troops (Horwich, 1983a). Between 1990 and 1995, three females successfully immigrated into the 10 troops. The full extent and ramification of both male and female migration will be resolved as more data are collected on the marked individuals. Based on models presented by Chesser (1991a,b), even a small amount of female migration would tend to homogenize gene frequencies among troops. It would have been interesting to study patterns of mtDNA among troops because as a maternally inherited marker, it might have provided additional data on the degree of female philopatry. Unfortunately, the low diversity of mtDNA made this molecule uninformative with regard to the study of population structure.

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